THE BIOCHEMISTRY AND PHYSIOLOGY OF BACTERIAL ADHESION TO SURFACES

Final Report to the Office of Naval Research

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by

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Abstract

The physiologic mechanisms involved in bacterial adhesion to inert surfaces have been investigated employing fouling isolates obtained from the Chesapeake Bay. Specifically, we have (1) compared the physiologic activity of attached and free-living bacteria (2) studied the effect of metabolic inhibitors on attachment to determine what physiologic processes were involved in adhesion (3) investigated the type of material employed as the adhesive substance.

We have developed two unique microfouling assays that employ the DNA-specific fluorochromes Hoechst 33258 and 33342. These two assays permit the sensitive and specific enumeration of attached populations of bacteria, and were used in the experiments described below.

Actively growing cells from log-phase cultures of Vibrio proteolytica adhered in greater numbers than late stationary phase or mercuric chloride-killed cells. Cells that were starved by resuspension in nutrient-free medium became progressively less adhesive.

We have measured the activity of attached and free-living cultures of V. proteolytica by rates of DNA synthesis employing ^{3}H-thymidine. Attached bacteria were more active than free-living bacteria during nutrient deprivation only. When peptone and yeast extract were added, free-living cells possessed greater activity than attached cells. Similarly, free-living natural populations of bacteria in the eutrophic Bayboro Harbor possessed greater activity than their attached counterparts.

The results of metabolic inhibitor studies imply that protein synthesis, RNA synthesis, and energy production were required in the adhesion process, whereas DNA synthesis was not. Since the sulfhydryl

blocking reagent p-chloromercuribenzoate and surfactants both inhibited attachment to polystyrene, it appeared that sulfhydryls and hydrophobic interactions were important in this attachment. Pronase treatment also prevented attachment and caused removal of previously attached cells.

Collectively, these results suggest that: 1) physiologically active cells are more adhesive than dead, starved, or physiologically impaired cells and 2) proteins and protein synthesis are involved in the adhesion of V. proteolytica to polystyrene, and the adhesive molecule itself may be a proteinaceous "adhesin".

Introduction

Much of the previous work on marine fouling has been limited to descriptive investigations of the fouling sequence. Only recently has attention been given to the molecular mechanisms of bacterial, algal, and invertebrate laval adhesion to surfaces in marine systems. Research into such areas, supported largely by a redirection in the Office of Naval Research's Biodeterioration program, is beginning to yield fundamental knowledge on cellular mechanisms of adhesion, and some of these findings can be found in this final report. Such knowledge, of great inherent value by itself, may aid in the development of unique antifoulants that would function by countering cellular adhesive mechanisms directly, being less toxic to shipyard personnel and marine life.

Over the past twelve months we have been investigating the physiology and biochemistry of bacterial adhesion to surfaces under ONR

contract #N00014-83-K-0024. In our research we have posed the following three questions:

1. Does attachment impart any physiologic advantage to cells? Are physiologically active cells more adhesive?

2. What are the biochemical and physiologic processes involved in adhesion? What effect will inhibitors of specific physiologic processes have on adhesion?

3. What is the nature of the adhesive material? Is it a collection of cell surface molecules, or is it comprised of exopolymers that are shed into the extracellular environment?

During this first year, we have developed a model system for bacterial adhesion employing Vibrio proteolytica and polystyrene as substratum (see attached publication). We have studied extensively the effect of metabolic inhibitors on this system (see attached manuscript), and most recently, we have started to elucidate the nature of the adhesive material employed by this organism. Additionally, we have set up a new microbiology lab and initiated a program in marine microbiology at USF.

After the successful development of our adhesion system and related techniques, we are now in a position to make rapid gains in the understanding of the adhesion of V. proteolytica, hopefully with the help of future funding from ONR.

Results of Research

Nine species of fouling bacteria have been previously isolated by our laboratory from the Chesapeake Bay (Paul and Loeb, 1983). We have developed two microfouling assays, one involving epifluorescence microscopy (Paul, 1982) and the other based on the fluorometric determination of DNA (Paul and Myers, 1982; Paul and Loeb, 1983). Employing these assays, it was found that the majority of our fouling isolates attached in greater numbers to polystyrene, a hydrophobic surface, than to glass, a hydrophilic surface (Paul and Loeb, 1983).

Our two most tenacious foulers, Vibrio proteolytica and Alteromonas citrea, have been studied in more detail. The results of this research is outlined below.

1. Does Adhesion Impart any Physiologic Advantages?

A widely accepted concept in fouling dogma is that non-metabolizable surfaces can increase bacterial activity, or that attached bacteria possess greater physiologic activity than unattached bacteria (Zobell, 1943; Jannasch and Pritchard, 1972) due to the adsorption of nutrients to surfaces (Marshall, 1976). Others have suggested bacteria adapt to nutrient deprivation by becoming attached (Dawson et al., 1981).

To answer this question, we have measured physiologic activity in attached and free-living bacteria by rates of DNA synthesis employing ^{3}H-thymidine (Fuhrman and Azam, 1982). Attached cells incubated in the absence of exogenous nutrients (in minimal salts medium) had greater rates of DNA synthesis than unattached cells (Fig. 1). In the presence

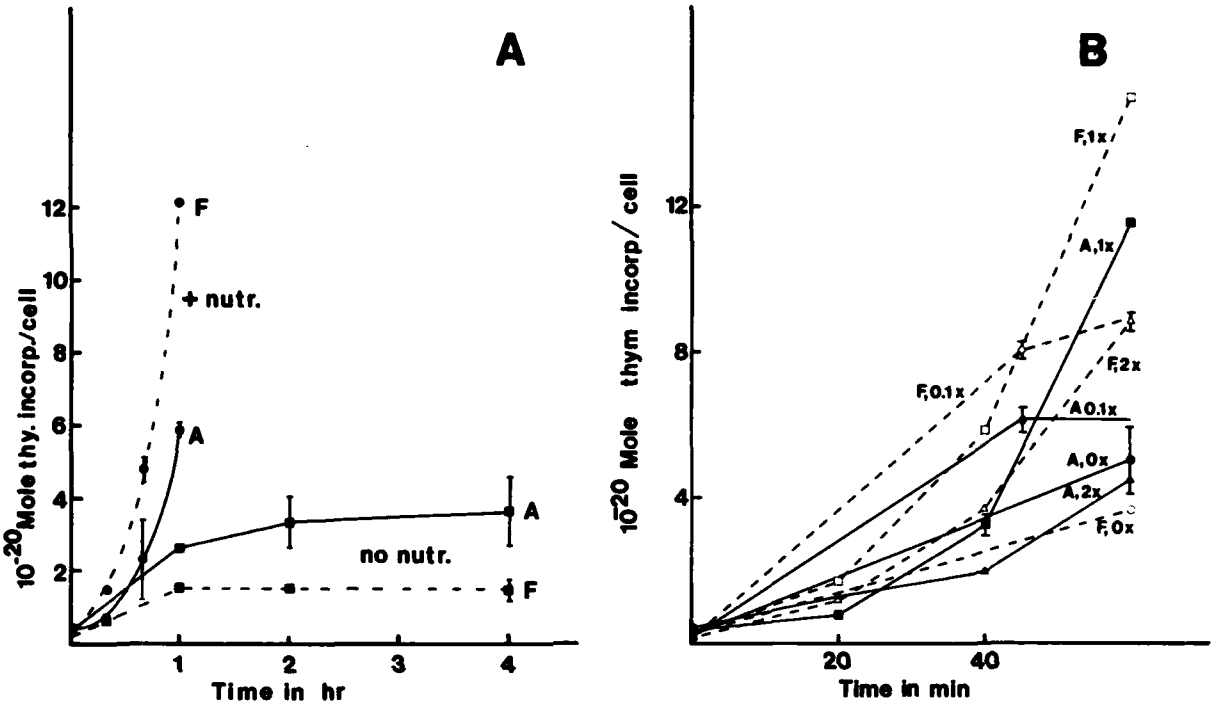

Figure 1. Incorportion of ^{3}H-thymidine into DNA in attached and free-living cells of Vibrio proteolytica. A) Incorporation of thymidine into DNA under nutrient concentration of growth (●; 0.167% peptone, 0.033% yeast extract, w/v) or nutrient deprivation (■, no peptone or yeast added). Total concentration of thymidine was 1 μM. Cell density for free-living cells (solid line) was 1.7×10^{6} cells/ml. Cell density on polystyrene dishes (labelled "A" for attached) was 1.6×10^{5} cells/mm^2. B) Incorporation of thymidine in attached and free-living cells at four nutrient concentrations. Nutrient concentrations employed were twice (labelled 2X, triangles), once (labelled 1X, squares), one-tenth (labelled 0.1, diamonds), or in the absence (0X, circles) of the normal nutrient concentration of growth (0.167% peptone, 0.033% yeast extract). Incorporation by free-living cells is signified by open symbols, dashed lines, and the letter "F". Attached cell incorporation is denoted by solid lines, filled symbols, and the letter "A". Initial free-living cell density was 5.8×10^{7} cells/ml, and attached cell density was 1.6×10^{5} cells/mm^2. Total thymidine concentration was 1 μM.

of various amounts of nutrients (peptone and yeast extract), unattached cells possessed greater rates of DNA synthesis than attached cells (Fig. 1a and 1b). An interpretation of these results is that during nutrient deprivation, any residual nutrients present may coat the surface, favoring growth of attached cells. When nutrients are in ample supply, rate of growth is limited by cell surface area available for nutrient transport or adsorption. Attached cells have a decreased surface area (losing the part in contact with the substratum) and thus cannot adsorb or transport nutrients as rapidly as unattached cells.

Rates of DNA synthesis have also been measured in natural populations of attached and free-living bacteria in Bayboro Harbor, a eutrophic, estuarine environment emptying into Tampa Bay. The rate of bacterial attachment appears in Fig. 2a. After ∿16hr the number of bacteria attached does not increase; barnacles then become prevalent. Our preliminary results (Fig. 2b) suggest that free-living bacteria were more active than those attached to polystyrene. Perhaps nutrient levels in Bayboro Harbor are so great that attached cells cannot compete for nutrients with free-living cells, as was found in high nutrient experiments with V. proteolytica.

We have also determined the adhesive capability of log, stationary, and nutrient-deprived cultures of V. proteolytica. Log phase cells were found to be more adhesive than stationary phase cells (data not shown). Starvation resulted in progressively less adhesive cells (Fig. 3a) which is in contrast to the findings of Dawson et al. (1981).

2. Which Physiologic Processes are Necessary in Adhesion?

Various metabolic inhibitors and antibiotics have been utilized to

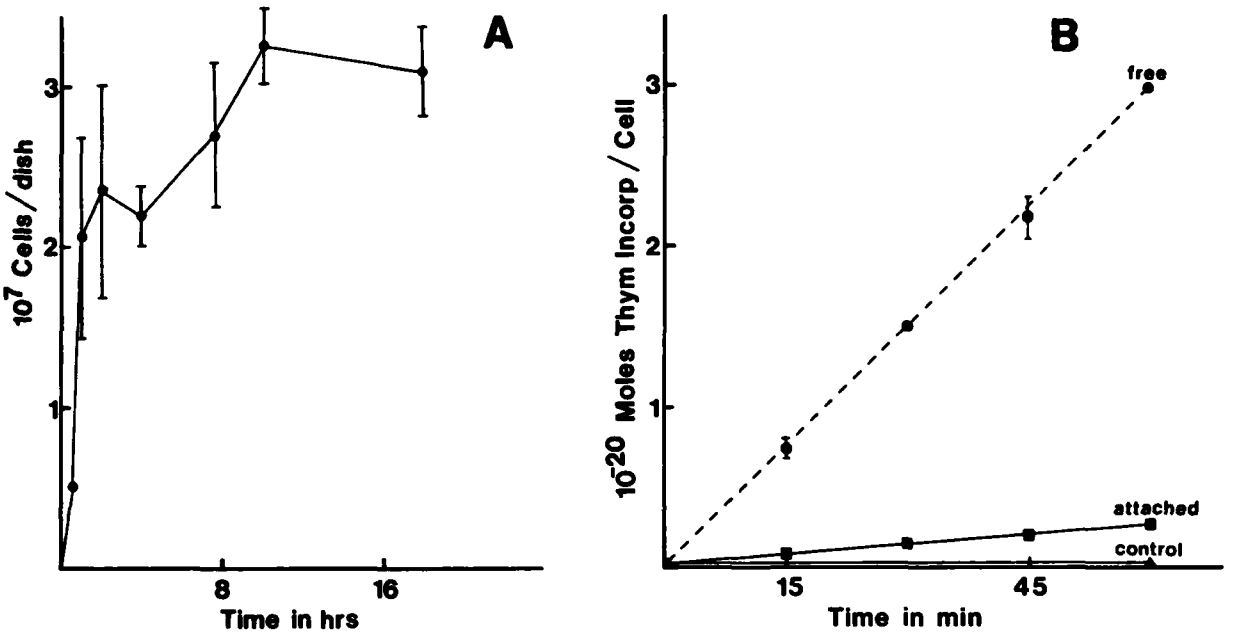

Figure 2. A. Attachment of natural bacterial populations to polystyrene culture dishes in Bayboro Harbor. Cells were enumerated by epifluorescence microscopy. Each point is the mean ± one standard deviation of three replicates.
B. Thymidine incorporation into DNA in natural attached and free-living microbial assemblages in Bayboro Harbor. Natural microbial assemblage was allowed to adhere for 3 hr in Bayboro Harbor. Free-living cells refer to DNA synthesis in a water sample collected at the time of collection of attached cells. Attached cell density was 2.8×10^3 cells/mm^2, free cell density was 6.8×10^6 cells/ml. Total unlabelled thymidine concentration was 5×10^{-9} M and a radioactivity of 0.4 μCi/ml was employed.

determine which physiologic processes are necessary for adhesion of V. proteolytica to polystyrene. Washed cell suspensions were presented simultaneously with the substratum and varying concentrations of the antimetabolites employed. The experimental design and results are described in detail in the attached manuscript. Inhibitors of DNA synthesis had little effect on attachment, while inhibitors of energy production, RNA and protein synthesis, and the sulfhydryl-blocking reagent p-chloromercuribenzoate all inhibited attachment. The extent of inhibition of attachment was less than the inhibition of growth. This effect may have been due to the temporal design of the experiments, and pretreatment with the inhibitor may have been necessary to produce maximal disruption of, say, adhesive synthesis. Adhesion assays employing washed cells that had been grown at 25% the minimal growth inhibiting concentration (MIC) indicated that the adhesion process was as sensitive to inhibitor treatment as growth (see attached manuscript).

Since the adhesion process was most sensitive to antimetabolites that affect proteins and aspects of protein synthesis, it seemed that proteins were involved in the adhesion process, whereas DNA synthesis (replication) was not.

3. The Nature of the Cell Adhesive

One approach to determining what type of material is employed as adhesive is to identify agents that dissolve or disrupt the cell-substratum bond. Since all isolates adhered in greater numbers to polystyrene, a non-wettable, hydrophobic substratum (Fletcher and Loeb, 1979) than to hydrophilic substrata, we attempted to disrupt this hydrophobic interaction with surfactants. The effects of two non-ionic

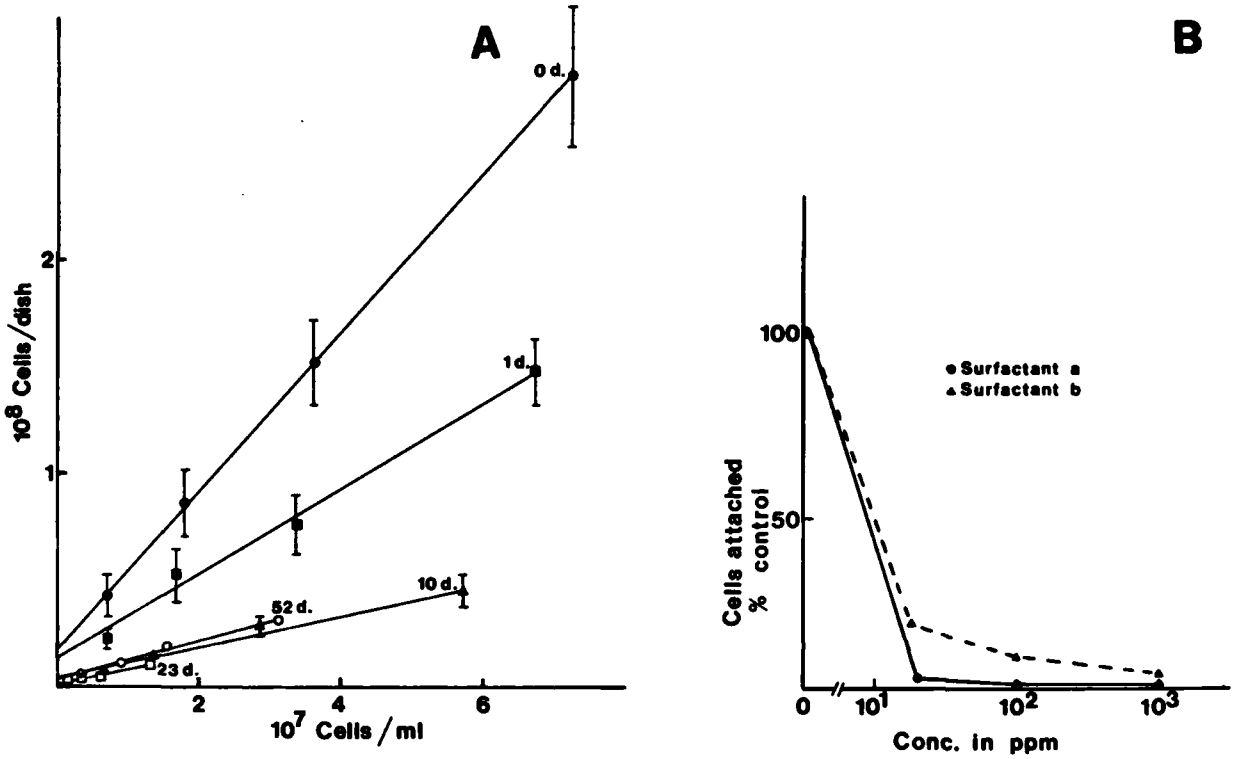

Figure 3A. Attachment of Vibrio proteolytica to polystyrene culture dishes as a function of culture cell density: Effect of starvation. A culture of V. proteolytica was harvested, washed and resuspended in minimal salts medium (30% ASWJP). Aliquots were taken as a function of time (labelled 0 to 23 'd.' for days), diluted, and allowed to adhere to polystyrene dishes for two hours. Each point is the mean ± one standard deviation of three replicates.

B. Attachment of Vibrio proteolytica to polystyrene in the presence of various concentrations of non-ionic surfactants. Surfactants were added to surfaces followed by the addition of cells. Two surfactants, A (Triton X-100) and B (Tween 80) were employed.

surfactants on the attachment of V. proteolytica to polystyrene appears in Figure 3b. Both surfactants strongly inhibited attachment, the greatest effect occurring with surfactant A. This surfactant also inhibited the attachment of all our other bacterial isolates to polystyrene (Table 1) and most were inhibited by concentrations as low as 20 ppm. Unlike metabolic inhibitors, surfactants completely inhibited attachment (95% inhibition).

V. proteolytica and A. citrea were examined by SEM for the presence of "bridging polymers" in the adhesion to polystyrene (Figures 4, 5, 6, 7). There was no evidence of such extracellular material (exopolymer) in V. proteolytica adhesion (Figs. 4, 5). On several occasions, bridging polymers were produced by A. citrea (Figs. 6, 7), although they were not always present.

Culture supernatants were analyzed for extracellular polysaccharide production by analysis for carbohydrate (Dubois et al., 1956) in cold ethanol precipitates. There was no net polysaccharide production by either organism, the culture medium containing at least as much ethanol precipitable carbohydrate. Extracellular polysaccharide and precipital uronic acid (Blumenkrantz and Asboe-Hansen, 1973) was found in Pseudomonas atlantica (Table 2), a known exopolymer producer which served as a control organism. A. citrea also produced some ethanol-precipitable uronic acid, which appeared in greatest quantities in late stationary or death culture phase (Table 2 and Fig. 8 and 9). Similar observations have been made for the production of a uronic acid polymer by Uhlinger and White (1983). The bridging polymers noted by SEM in A. citrea may be the ethanol-precipitable uronic acid noted in culture supernatants.

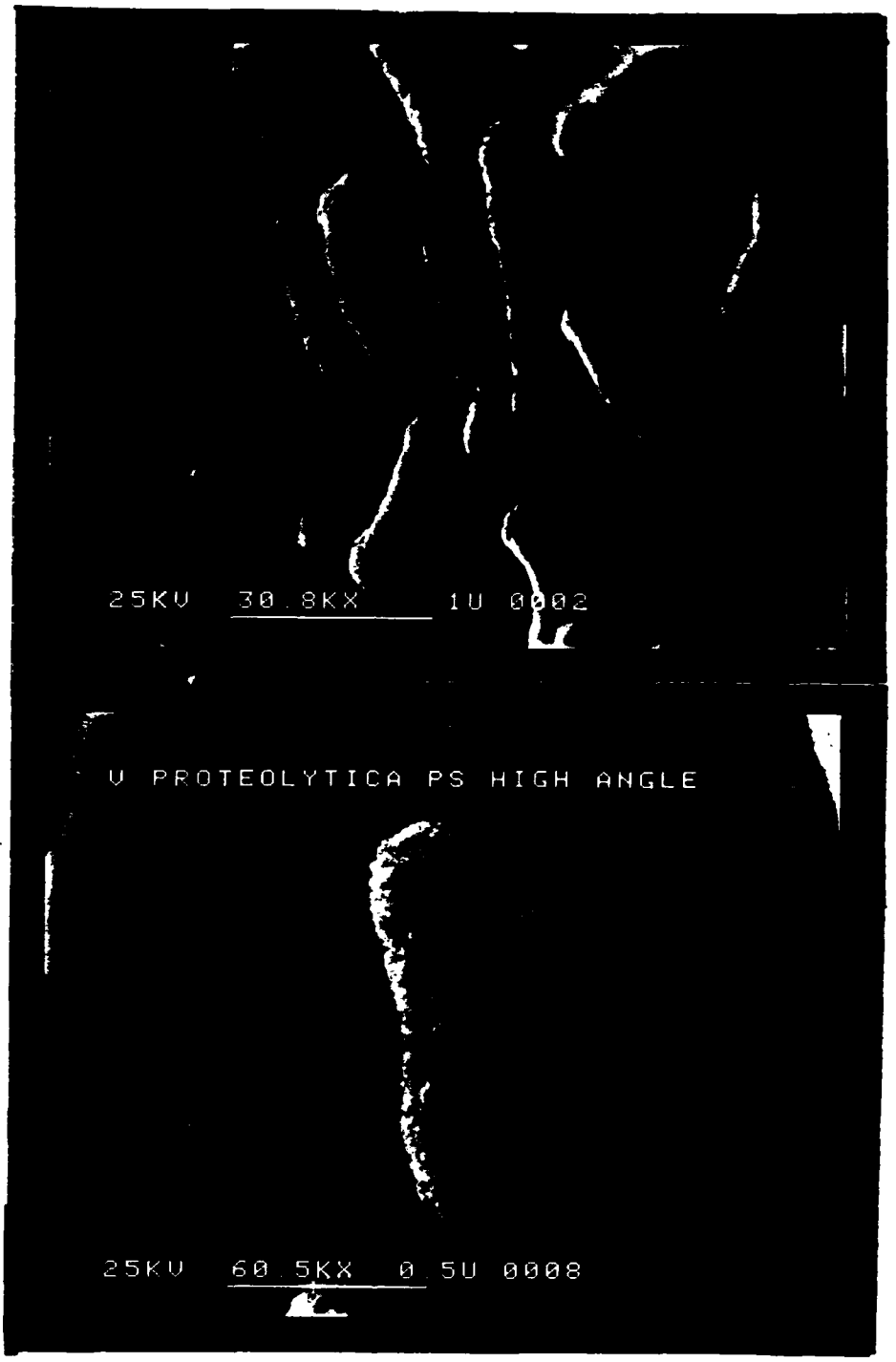

Figure 4A & B. Scanning Electron Microscopy (SEM) of *Vibrio proteolytica* cells adhered to polystyrene. Note absence of cell surface "Bridging Polymers" or exopolymers.

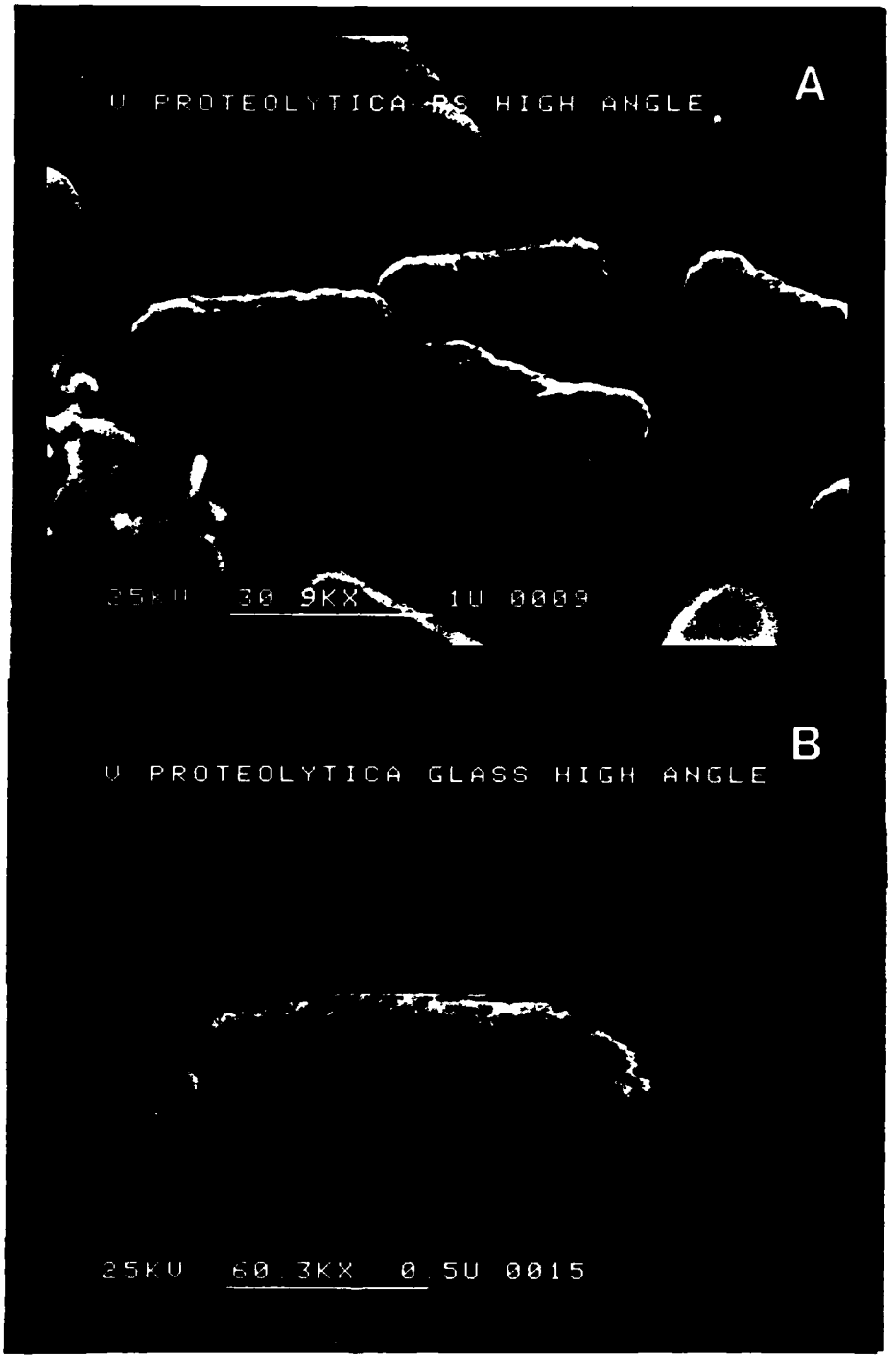

Figure 5. SEM of V. proteolytica attached to Polystyrene (A) or Glass (B).

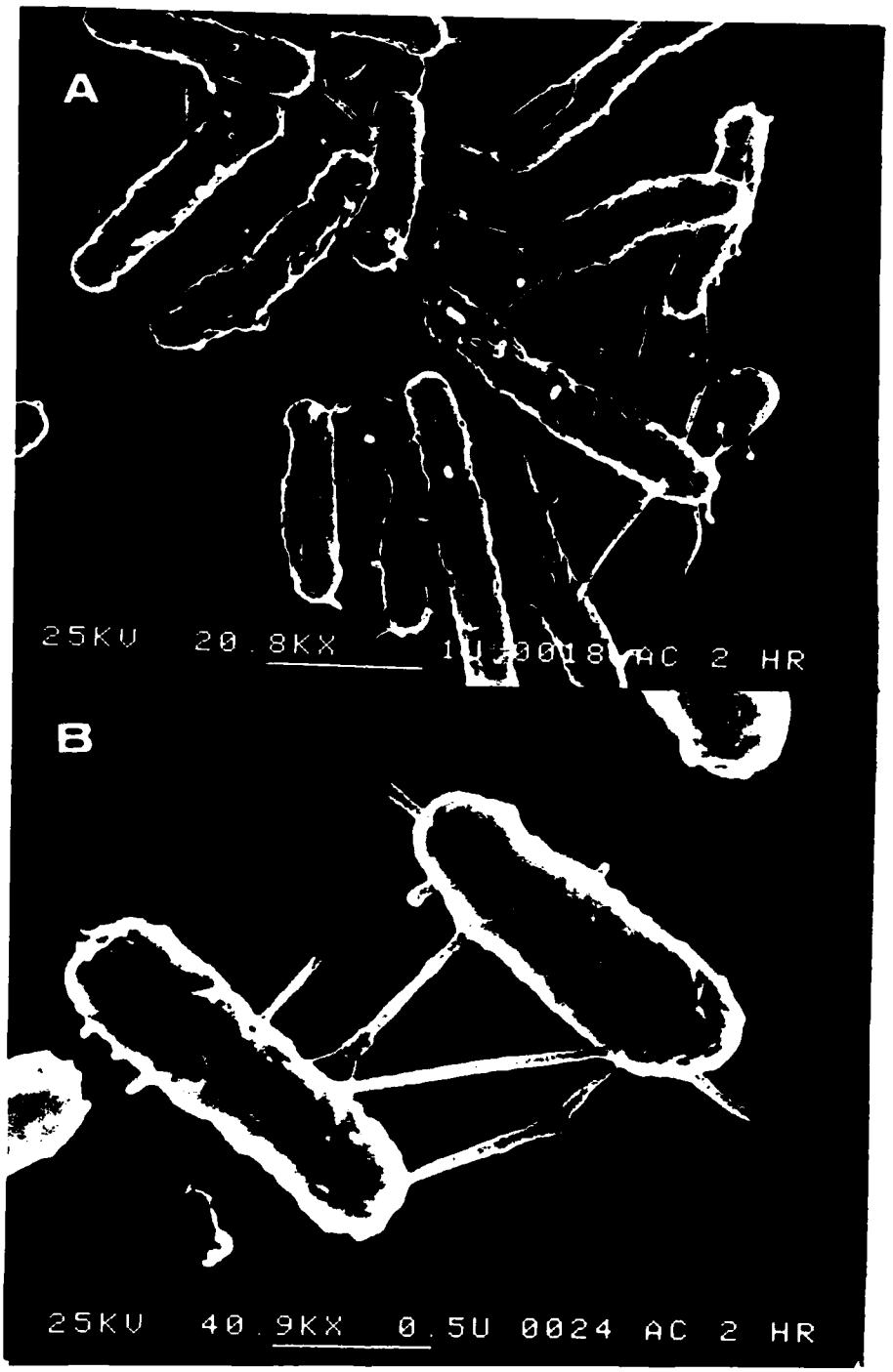

Figure 6A & B. Attachment of Alteromonas citrea to polystyrene. Note the production and involvment of copious amounts of exopolymer or "bridging polymers" in the adhesion process.

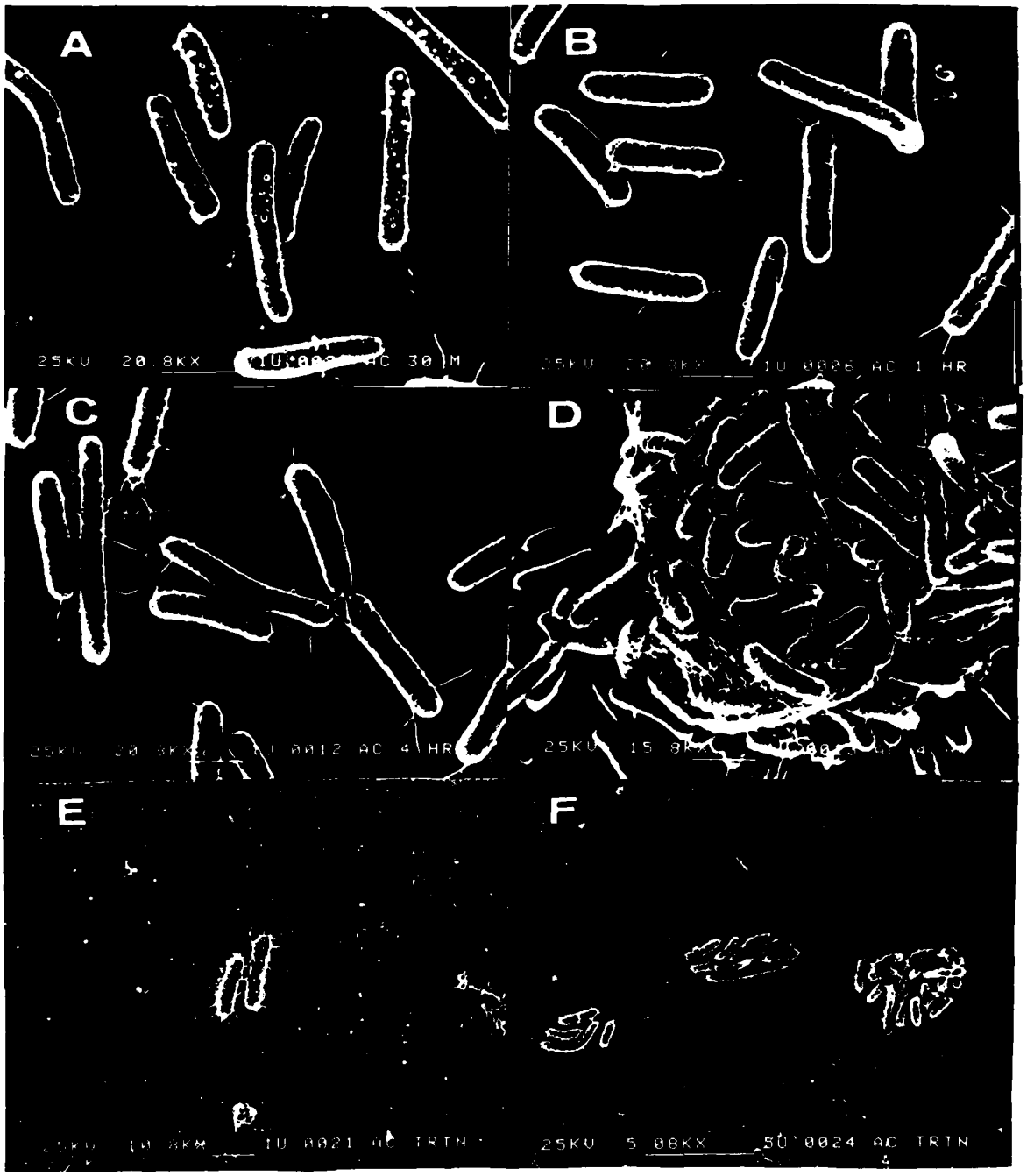

Figure 7. Attachment of A. citrea to polystyrene. Note involvement of flagella (Figs a-c) and the mixed-mass of flagella and exopolymer. Fig 7E,F. Alteromonas citrea attached to polystyrene after surfactant treatment (0.002% surfactant A).

Since inhibitor studies implied proteins in the adhesion process and since no extracellular polysaccharide was produced by V. proteolytica, we investigated the effect of pronase (protease from Streptomyces griseus) on attachment (Fig. 10a, b). When administered simultaneously with the substratum, protease strongly inhibited attachment, with complete inhibition (98%) occurring at concentrations of 100 and 250 $\mu g\ ml^{-1}$. Boiled protease stimulated attachment at 20 and 50 $\mu g\ ml^{-1}$, while lower and higher concentrations slightly inhibited attachment (Fig. 10a). Protease treatment did not cause cell lysis. Protease treatment could remove cells that had attached for 2 hr, leaving behind what appeared as faint outlines ("footprints") of the previously attached cells, suggesting breakage of the bond between cell and polymer.

Discussion: Significance of Results

The results of studies with V. proteolytica indicate that physiologically competent cells can adhere better than dead, physiologically impaired, or starved cells. Thus adhesion in this organism appears to be a physiologically modulated process, and more than just the result of physiochemical interactions. Adhesion does not seem to be a response to starvation as found for a Vibrio by Dawson et al. (1981).

Adhesion does not impart a physiologic advantage to V. proteolytica cells, unless the cells are in a nutrient-deprived condition. Otherwise, adhesion may result in slower growth, due to the unavailability for nutrient uptake of the portion of the cell's surface

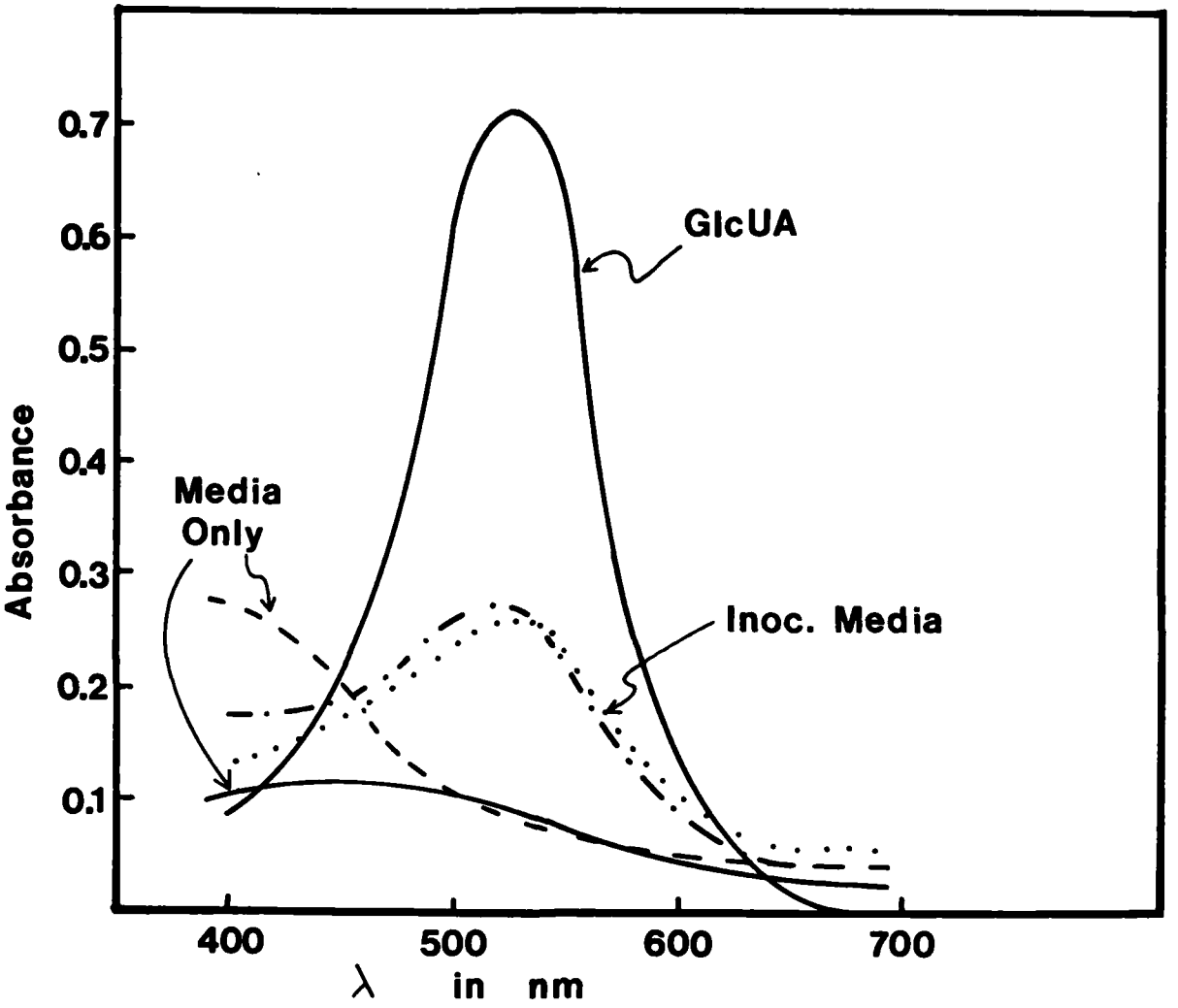

Figure 8. Adsorption spectra of chromophore formed between uronic acids and M-OH diphenyl in sulfuric acid/sodium tetraborate (method of Blumerkrantz and Asboe-Hansen, 1973). Curve labelled GlcUA is glucuronic acid, 20 µg. The ethanol precipitable polysaccharide from spent growth media from Alteromonas citrea (labelled "inoc media") and uninoculated media (labelled "media only") were also tested for uronic acid content by this technique. Apparently A. citrea produces a slight amount of a uronic acid polymer.

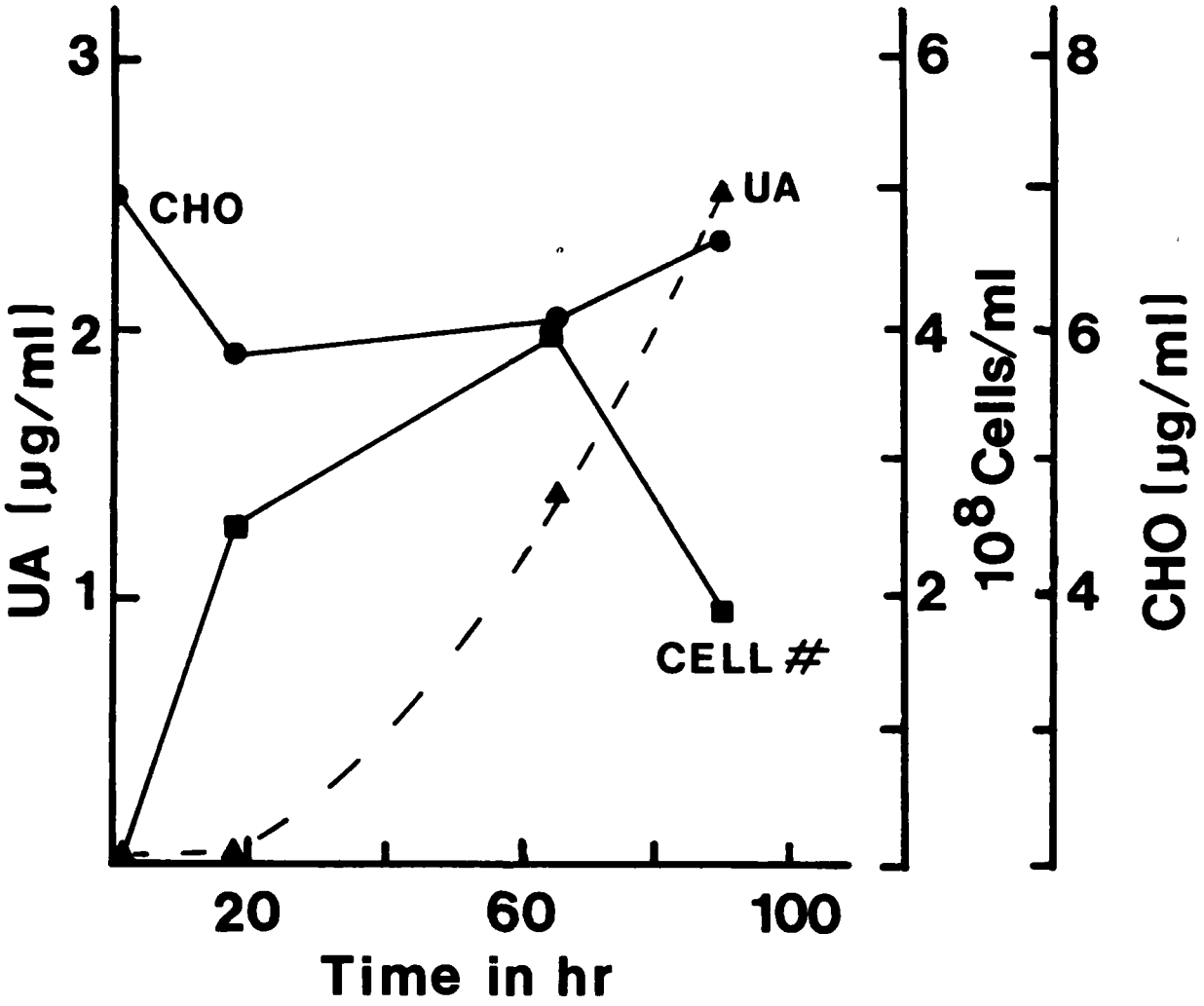

Figure 9. Ethanol-precipitable carbohydrate (●), and uronic acid (▲), and cell density (■) in cultures of A. citrea as a function of time.

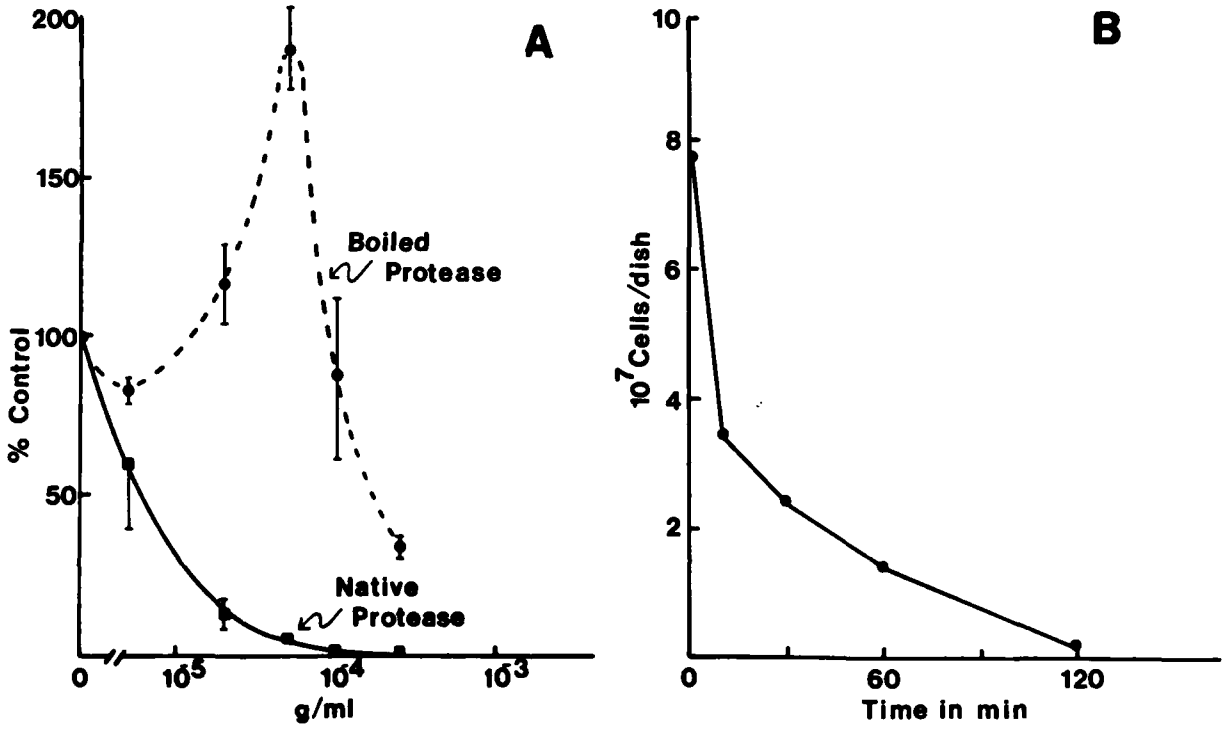

Figure 10. The effect of Pronase (Protease from Streptomyces griseus) on the attachment of V. proteolytica to polystyrene. A). The effect of various concentrations of native or boiled (40 min) protease on attachment when adminstered to substratum simultaneously with cells. Incubation time was 2 hr.
B). Removal of cells previously attached to polystyrene by pronase (100 $\mu g\ ml^{-1}$). After cells were removed by this treatment, faint outlines or "ghosts" could be observed on the polystyrene.

that is in contact with the substratum. The lack of exopolymer produced by this organism casts doubt on its use for trapping nutrients (Geesey et al., 1982; Costerton and Geesey, 1979). This lack of exopolymer suggests that molecules firmly bound to the surface of the cell or the outer layers of the wall itself may be the adhesive moiety. The mechanism of attachment of this organism is quite different from the extracellular acidic polysaccharides employed by Pseudomonas atlantica (Corpe, 1970) and other marine bacteria (Jones et al., 1969; Fletcher and Floodgate, 1973; Geesey et al., 1977; Uhlinger and White, 1983). We term this latter type of adhesive mechanism the Pseudomonas type adhesion.

Since protein synthesis inhibitors, RNA synthesis inhibitors, sulfhydryl blocking reagents, and proteolytic enzymes all inhibited attachment by V. proteolytica, it seems that proteins are the adhesive molecule. These proteins may be similar to the "adhesins" employed by enterotoxigenic E. coli strains to adhere to intestinal epithelium (Vosbeck et al., 1979; 1982; Lindahl et al., 1981). Similar adhesins are believed responsible for haemagglutination in aeromonads and Vibrios (Jiwa, 1983) and may have lectin-like properties (Faris et al., 1982). Such molecules also cause cell surface hydrophobicity, an attribute of V. proteolytica.

In conclusion, we may be on the verge of describing a unique bacterial adhesive mechanism previously unreported in marine bacteria, but common in enterotoxigenic bacteria. This mechanism may explain the widespread preference for hydrophobic surfaces observed in marine bacteria. This adhesive mechanism is apparently totally different from the acidic polysaccharide/exopolymer mechanism which has been widely proselytized as the major mechanism of marine bacterial adhesion.

Table 1. Effect of Surfactant A on Attachment of Isolates to Polystyrene.

Isolate	Cells/Dish	% Control
1 Control	$1.34 \pm 0.22 \times 10^8$	100
0.002% Surfactant A	$2.09 \pm 0.84 \times 10^6$	1.6
2 Control	$2.69 \pm 0.61 \times 10^7$	100
0.002% Surfactant A	$4.64 \pm 3.48 \times 10^5$	1.72
3 Control	$1.29 \pm 0.16 \times 10^8$	100
0.002% Surfactant A	$1.3 \pm 0.33 \times 10^5$	0.1
4 Control	$5.9 \pm 1.47 \times 10^5$	100
0.002% Surfactant A	ND [a]	<<1
5 Control	$1.38 \pm 0.23 \times 10^8$	100
0.002% Surfactant A	$3.58 \pm 2.1 \times 10^5$	0.26
7 Control	$2.31 \pm 1.1 \times 10^6$	100
0.002% Surfactant A	1.13×10^5	4.9
8 Control	$7.78 \pm 0.9 \times 10^7$	100
0.002% Surfactant A	$2.33 \pm 0.4 \times 10^6$	3
9 Control	$6.51 \pm 0.53 \times 10^7$	100
0.002% Surfactant A	$2.15 \pm 0.96 \times 10^7$	33
0.01% Surfactant A	5.73×10^4	0.08
0.1% Surfactant A	ND	<<1

[a] ND means cell density too dilute to count.

Table 2. Polysaccharide Content of Culture Supernatants

Sample	Carbohydrate µg/ml	Uronic Acid µg/ml	10^8 Cells/ml	A_{600} of Culture
ASWJP 30% Media with Peptone, Yeast extract				
Media only	8.6±2.2	1.22±0.4	----	----
Vibrio proteolytica	9.7±1.0	1.6±0.31	9.5	0.95
Alteromonas citrea	8.7±0.8	2.48±0.6**	4.5	0.70
ASWJP 30% Media with Asparagine, Sucrose				
Media only	3.67±1.6	0.95±0.4	----	----
Vibrio proteolytica	5.0±1.0	0.9±0.1	2.72	1.03
Alteromonas citrea	4.1±0.5	2.55±1.5	0.43	0.79
ASWJP 100% Media - Net Polysaccharide Production				
Pseudomonas atlantica	14.1±1.4**	6.5±0.12**	14.0	----

**Carbohydrate or Uronic acid content significantly greater than media alone

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EFFECTS OF ANTIMETABOLITES ON THE ATTACHMENT OF AN ESTUARINE *VIBRIO* TO POLYSTYRENE

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Abbreviations: MIC, minimal inhibitor concentration.

ABSTRACT

The effect of various antimetabolites (metabolic inhibitors and antibiotics) on the growth and attachment of Vibrio proteolytica to polystyrene has been investigated. Inhibitors of protein synthesis (puromycin and chloramphenicol), RNA synthesis (6-azauridine and rifampicin), energy production (azide and 2,4-dinitrophenol), and the sulfhydryl blocking reagent p-chloromercuribenzoate all inhibited attachment at high concentrations when administered simultaneously with the substratum. The extent of inhibition of growth by these compounds was greater and occurred at lower concentrations than inhibition of attachment. The DNA synthesis inhibitors nalidixic acid and 5-fluorodeoxuridine and the protein synthesis inhibitor streptomycin sulfate all inhibited growth while having little effect on attachment. After growth at the 25% minimal inhibitory concentration of the above compounds, the magnitude of the inhibition of attachment was approximately the same as that for growth. The greatest departure from this occurred with streptomycin sulfate, which under these conditions inhibited attachment by ∿70% and growth by ∿38%. These results suggest that 1) proteins, protein synthesis (including transcription) and energy production are required in the attachment of Vibrio proteolytica to polystyrene and 2) growth at sub-MIC of metabolic inhibitors is a better means of assessing the susceptibility of the attachment process to these compounds.

INTRODUCTION

The attachment of bacteria to inert surfaces often is regarded as a physiochemical process, governed by the surface properties of the cell and the substratum (Tadros 1980). Models describing cell attachment have been based upon the Langmuir isotherm equation for the adsorption of gas molecules to a surface (Fletcher 1977, Belas and Colwell 1981), a thermodynamic consideration of the surface tensions (γ) of the bacteria, fluid, and substratum (Gerson 1981; Absolom et al. 1983), and the Michaelis-Menten equation of enzyme kinetics (Cohen et al. 1981).

The physiochemical properties of a cell's surface (hydrophobicity, hydrophilicity, surface charge, etc.) which determine its adhesive characteristics are in turn determined by cell surface macromolecules and the cell's capacity to synthesize these macromolecules. Thus physiologic processes also play a role in bacterial attachment to inert surfaces. For example, log phase cells have been found to adhere in greater numbers than stationary or death phase cells (Fletcher 1977) and the energy production inhibitors potassium cyanide, oligomycin, malonate and dinitrophenol have inhibited the rapid attachment (occurring in minutes) of a marine pseudomonad to polystyrene (Fletcher 1980).

In an effort to determine what physiologic processes may be necessary for adhesion, we have investigated the effect of various metabolic inhibitors (protein, DNA, RNA, and cell wall synthesis inhibitors, and energy production inhibitors) on the adhesion of an estuarine Vibrio to polystyrene. Others investigating the effect of antimetabolites on bacterial attachment have employed concentrations well in excess of those required to inhibit the specific physiologic process (Fletcher 1980), and thus effects observed may have been due to

secondary effects of the inhibitor. To avoid this, we have investigated the effect of a range of concentrations of inhibitors on cell attachment as well as attachment after growth on 25% of the minimal inhibitory concentration (MIC) of these inhibitors.

MATERIALS AND METHODS

Antimetabolites. 5-Fluorodeoxyuridine, p-chloromercuribenzoic acid, nalidixic acid, 2,4-dinitrophenol, 6-azauridine, cephalosporin C, streptomycin sulfate, chloramphenicol, rifampicin, sodium azide, and puromycin were obtained from Sigma Chemical Co., St. Louis, Missouri, and were the finest grade available.

Organism and Growth Conditions. Vibrio proteolytica was isolated from polyvinyl chloride coverslips suspended in the Chesapeake Bay as described previously (Paul and Loeb 1983). Stock cultures were maintained at 15° on an artificial seawater agar enriched with peptone and yeast extract (Paul and Loeb 1983). For adhesion assays, cells were grown in 50 ml batches of a mineral salts medium containing 0.17% (w/v) peptone and 0.033% yeast extract (ASWJP, Paul 1982) at 15° and harvested after 16 hr ($1 - 2 \times 10^9$ cells/ml) by centrifugation at 10,000 x G. Cell pellets were washed twice with ASWJP medium lacking peptone and yeast extract (ASWJP-PY) and added to 15 x 60 mm polystyrene dishes (Falcon, Oxnard, California) which contained the appropriate concentration of inhibitor or antibiotic, for a final cell density of $2 - 5 \times 10^7$ cells ml^{-1}. Dishes were incubated for 2 hr at 15°, washed by dipping three to four times through three washes of ASWJP-YE, and cell density determined by epifluorescent direct counts after staining with Hoechst 33258 (Paul 1982). For attachment as a function of time,

dishes were treated as above but cell density estimated as DNA content (Paul and Loeb 1983). For growth studies, 1 ml inoculum (1 - 2 x 10^9 cells/ml) was added to 50 ml aliquots of ASWJP containing various concentrations of the inhibitors to be investigated. Cultures were incubated for 16-18 hr, and cell density determined by absorbance at 600 nm.

Attachment after Growth at 25% MIC of Antimetabolites. The MIC (Vosbeck et al. 1979) of the various antimetabolites was determined from the above growth studies. Fifty ml ASWJP containing 25% MIC of a particular antimetabolite was inoculated with 1 ml bacterial suspension containing 1 - 2 x 10^9 cells/ml. After 16-18 hr incubation at 15°, cells were harvested, washed, and resuspended at a cell density corresponding to an A_{600} of 0.2 (2 - 4 x 10^7 cells/ml) in ASWJP-PY. An aliquot of this suspension was taken for direct counts and 2 ml portions were added to 6.0 ml ASWJP-PY in polystyrene dishes. After a 1 hr attachment period, dishes were washed, stained, fixed, and counted as before (Paul 1982).

Procedures for Scanning Electron Microscopy (SEM). Four by four mm squares were cut from the bottom of polystyrene dishes and washed in 95% ethanol with minimal handling. Squares were placed in suspensions of cells prepared for adhesion assays as described above. After the adhesion period, polystyrene squares were rinsed three times in ASWJP-PY and fixed for 1 hr in 4% glutaraldehyde in ASWJP-PY. The pieces were then washed in deionized water, dehydrated by immersion in increasing concentrations of ethanol (10-15 min at each concentration), and critical point dried in CO_2. Electron photomicrographs were taken with a DS-130 SEM (International Scientific Instruments, Santa Clara, California) employing the top (high resolution) stage.

RESULTS

The attachment of Vibrio proteolytica to polystyrene in the presence of 50 ppm $HgCl_2$ appears in Figure 1A. Mercuric chloride inhibited attachment in the first minutes of the experiment, and inhibition continued throughout the duration of the experiment. The effect of the energy production inhibitors 2,4-dinitrophenol and sodium azide on attachment appears in Fig. 1B and C, respectively. Both inhibited attachment at the highest concentration employed, the greatest inhibition occurring with dinitrophenol. Growth was inhibited to a greater extent than attachment for both inhibitors, the largest difference occurring for azide. During growth on subminimal inhibitory concentrations, Vibrio proteolytica converted the yellow dinitrophenol to an unidentified brownish product.

The sulfhydryl blocking reagent p-chloromercuribenzoate also inhibited attachment at concentrations in excess of those required to inhibit growth (Fig. 1D).

The effect of protein synthesis inhibitors on attachment appears in Figures 2A, B, and D, and attachment in the presence of the cell wall synthesis inhibitor cephalosporin C appears in Figure 2C. Chloramphenicol and puromycin both inhibited attachment at high concentrations, chloramphenicol producing the greater effect. Streptomycin, when administered simultaneously with the substratum, had little effect on attachment, while greatly inhibiting growth. Cephalosporin C only slightly inhibited growth and had no detectable effect on attachment.

The results of simultaneous presentation of substrata and nucleic acid synthesis inhibitors appear in Figure 3. The RNA synthesis

inhibitors rifampicin and 6-azauridine both inhibited attachment at elevated concentrations, rifampicin having the greater effect. Although the DNA synthesis inhibitors nalidixic acid and 5-fluorodeoxyuridine both inhibited growth, neither inhibited attachment, and fluorodeoxyuridine may have stimulated attachment (Fig. 3B).

To elicit the greatest effect of these inhibitors on attachment, preincubation or growth in the presence of these inhibitors may have been necessary. It is conceivable that simultaneous presentation of inhibitor with substratum might not have any effect on adhesive polymers previously synthesized. Therefore, cells were grown at 25% MIC of the inhibitors, resuspended at a cell density as control cells, and presented the substrata in the absence of the inhibitor (Table 1).

The magnitude of the effect of the inhibitors on attachment under these conditions was similar to that of growth. In general, antimetabolites that inhibited attachment at ten times the MIC also inhibited attachment after growth at 25% MIC. The largest departure from this occurred with streptomycin sulfate, which had the greatest effect on attachment ($\sim$70% inhibition). Other anomalies included the slight but significant ($\alpha = 0.05$, $p>0.01$) inhibition of attachment by nalidixic acid, and the lack of effect of azauridine after growth at 25% MIC. This experiment could not be performed with cephalosporin C since no concentration employed completely inhibited growth, and a MIC could not be determined.

Due to the pronounced effect of streptomycin on attachment after growth at 25% MIC, we looked for morphologic variations by SEM in cells grown in the presence of streptomycin (Fig. 4). After growth on 25% MIC streptomycin, cell morphology was distorted and the cell surface

appeared convoluted (Fig. 4C-E) when compared to the smooth, featureless control cells (Fig. 4A & B). No evidence of bridging polymers was found in control cells, but several streptomycin-treated cells displayed some extracellular material that may have had adhesive properties.

DISCUSSION

From the above results, it appears that proteins and protein synthesis are necessary for adhesion by Vibrio proteolytica to polystyrene, while DNA synthesis is not. Cephalosporin C, which prevents the final transpeptidation step in bacterial cell wall pentapeptide synthesis, had little effect on adhesion. It is not known if growth at a sub MIC of cephalosporin C would have affected attachment, since a MIC could not be determined.

That mercuric chloride-killed cells adhered in lower numbers than control cells indicates that cell viability aids in the adhesion process. The attachment of nonliving cells has been termed "passive adsorption" to be distinguished from "active adsorption", or that which is dependent upon physiologic processes (Fletcher 1980). The requirement for viability is apparently manifested in the first minutes of attachment.

The requirement for ATP production is suggested by the effects of the electron transport inhibitors and uncouplers, azide and dinitrophenol. This requirement for ATP may be for motility or perhaps protein synthesis.

The effect of 6-azauridine, an inhibitor of orotic monophosphate decarboxylase (Hochster and Quastel 1963) on attachment was less than the RNA polymerase inhibitor rifampicin. Perhaps endogenous nucleotide

pools were sufficient to support RNA synthesis during simultaneous exposure to azauridine and the substratum. During growth at sub MIC concentrations of azauridine, perhaps the pyrimidine content of the medium (which contained peptone and yeast extract) was sufficient to meet nucleotide precursor requirements.

Puromycin, which terminates polypeptide chain synthesis by its incorporation into growing peptides, and chloramphenicol, a peptidal transferase inhibitor which binds to the 50S ribosomal subunit, both prevent polypeptide chain elongation (Hahn 1967, Fernandez-Munoz et al. 1972). Fletcher (1980) also found these inhibitors to impair the attachment of several marine bacteria to polystyrene. If peptide elongation is required in adhesion, it is disconcerting that streptomycin, an inhibitor of the initiation, elongation, and termination of peptide synthesis (Bollen et al. 1972), had little effect on attachment when simultaneously administered with the substratum. Net protein synthesis ceases within 5-15 min of the administration of streptomycin (Jacoby and Gorini 1967). Streptomycin apparently affected attachment in actively growing cells only.

Vosbeck et al. (1982) found that growth in the presence of 25% MIC of certain protein synthesis inhibitors (streptomycin, chloramphenicol, tetracycline) reduced the adhesiveness of several E. coli strains to intestine monolayer cells. The DNA synthesis inhibitor nalidixic acid made these cells more adhesive. Protein factors or "adhesins" were implied in this adhesion (Vosbeck et al. 1979, 1982).

Extracellular acidic polysaccharides ("glycocalyces") have been implicated in many instances of marine bacterial adhesion (Jones et al. 1969; Corpe 1970; Fletcher and Floodgate 1973; Geese et al. 1977; Fazio

et al. 1982; Uhlinger and White 1983). It is conceivable that the effects of protein and RNA synthesis inhibitors on adhesion may have been due to impaired extracellular polysaccharide biosynthesis, which in turn inhibited attachment. Perhaps a sulfhydryl-enzyme was required in this polysaccharide synthesis. However, we have not observed any extracellular material in the adhesion of Vibrio proteolytica to polystyrene by SEM and epifluorescence microscopy, although we have noticed "bridging polymers" in the attachment of Alteromonas citrea (unpublished results). Additionally, chloramphenicol purportedly allows polysaccharide (as well as DNA and cell wall) biosynthesis to proceed unimpaired (Hahn 1967) while only inhibiting protein synthesis. Lastly, this organism prefers hydrophobic substrata (Paul and Loeb 1983) and is dislodged by surfactants (J.H. Paul, unpublished data), suggesting that hydrophobic interactions are important in this adhesion. In general, hydrophobic strains are not encapsulated, since glycocalyces and capsules make bacteria hydrophilic (Hogt et al. 1982).

The nature of the hydrophobic moiety at the cell surface of bacteria attaching by hydrophobic interactions is not known (Marshall and Cruikshank 1973). Proteins have been implied in some of these interactions since pepsin treatment decreased the capacity for hydrophobic attachment to FEP-teflon (Hogt et al. 1982) and pronase and trypsin treatment inhibited attachment of a marine Pseudomonas to polystyrene (Fletcher and Marshall 1982).

These hydrophobic moeities may be similar to those employed by enterotoxigenic E. coli species to attach to mucosal epithelial surfaces (Lindahl et al., 1981). These are protein "adhesins" which are surface antigens that form hairlike structures (pili or fimbrae). These

adhesins are believed responsible for haemagglutination in aeromonads and Vibrios (Jiwa 1983) and may have lectin-like properties based on their interaction with mannose (Faris et al. 1982). This type of hydrophobic attachment may be completely different from that hypothesized by Moore & Marshall (1981), who described hydrophobic interactions as those responsible for reversible adhesion only. Similar proteins may be involved in the attachment of Vibrio proteolytica to polystyrene.

ACKNOWLEDGEMENTS

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Table 1. Effect of 25% Subminimal Inhibitory Concentrations of Antimetabolites on Growth and Attachment of Vibrio proteolytica. Cells were grown at the 25% MIC of the appropriate antibiotic, growth measured, harvested and washed twice, and resuspended at an identical cell density as controls for attachment to polystyrene. Attachment occurred in the absence of antibiotics.

Treatment	25% MIC	Growth (% Control)	Attachment
None	---	100.0	100.0
Dinitrophenol	1.2×10^{-4} M	80.8	91.9
Sodium Azide	7.5 μg ml^{-1}	56.5	58.4
Streptomycin sulfate	2.5 μg ml^{-1}	61.5	32.4
Chloramphenicol	0.3 μg ml^{-1}	47.2	75.7
Puromycin	6.25×10^{-5} M	80.4	75.1
p-Chloromercuribenzoate	1.25×10^{-5} M	38.7	71.7
Fluorodeoxuridine	1.5×10^{-5} M	100.0	136.0
Nalidixic acid	2.5×10^{-6} M	99.7	88.2
Azauridine	1.5×10^{-4} M	50.8	100.0
Rifampicin	0.125 μg l^{-1}	86.9	87.3

FIGURE LEGENDS

Figure 1A. Effect of 50 ppm $HgCl_2$ on the rate of attachment of Vibrio proteolytica to polystyrene. Cells were treated with $HgCl_2$ 15 min prior to and during exposure to polystyrene dishes. Cell density is expressed as DNA content.

B-D. The effect of metabolic inhibitors and antimetabolites on the growth and attachment of Vibrio proteolytica to polystyrene. 'A' denotes attachment, 'G' denotes growth. B, 2,4-Dinitrophenol, C, sodium azide, D, p-chloromercuribenzoate.

Figure 2. The effect of antimetabolites and metabolic inhibitors on the attachment and growth of Vibrio proteolytica. All values are expressed as % control; 'A' represents attached cells, 'G' represents cell growth. A, puromycin, B, streptomycin, C, cephalosporin C, D, chloramphenicol.

Figure 3. The effect of RNA synthesis inhibitors (Rifampicin, A, and Azauridine, C) and DNA synthesis inhibitors (5-Fluorodeoxyuridine, B, and Nalidixic acid, D) on growth and attachment of Vibrio proteolytica to polystyrene, sympols as in Fig. 1.

Figure 4. Scanning electron microphotographs of Vibrio proteolytica attached to polystyrene. Scale is indicated by the bar, and U = μm. A, B, Control cells; C-F, cells grown at 25% MIC streptomycin sulfate. Notice cell surface deformation and unusual morphology of streptomycin-treated cells, as compared to controls.

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Fig 1.

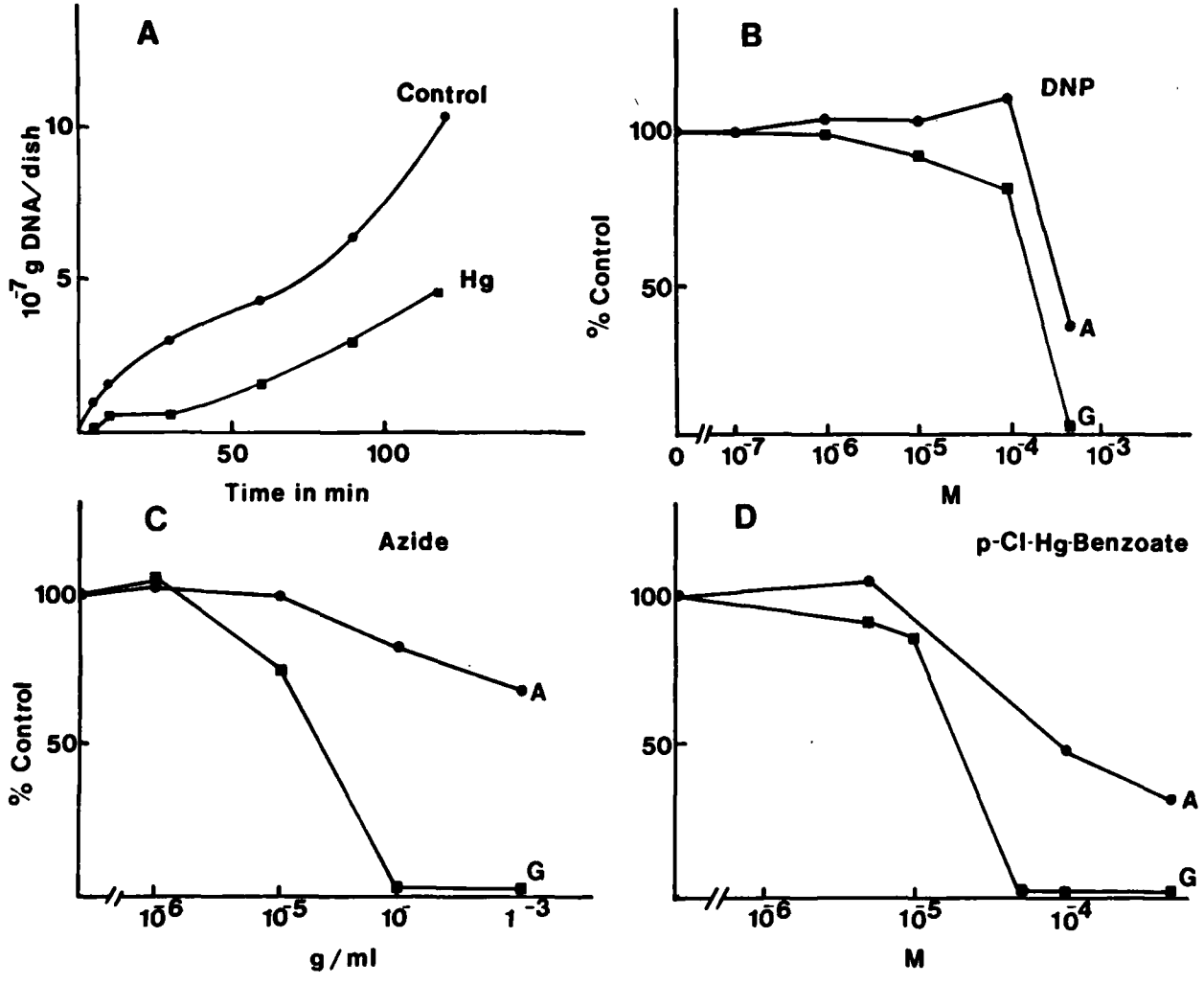
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Fig 2

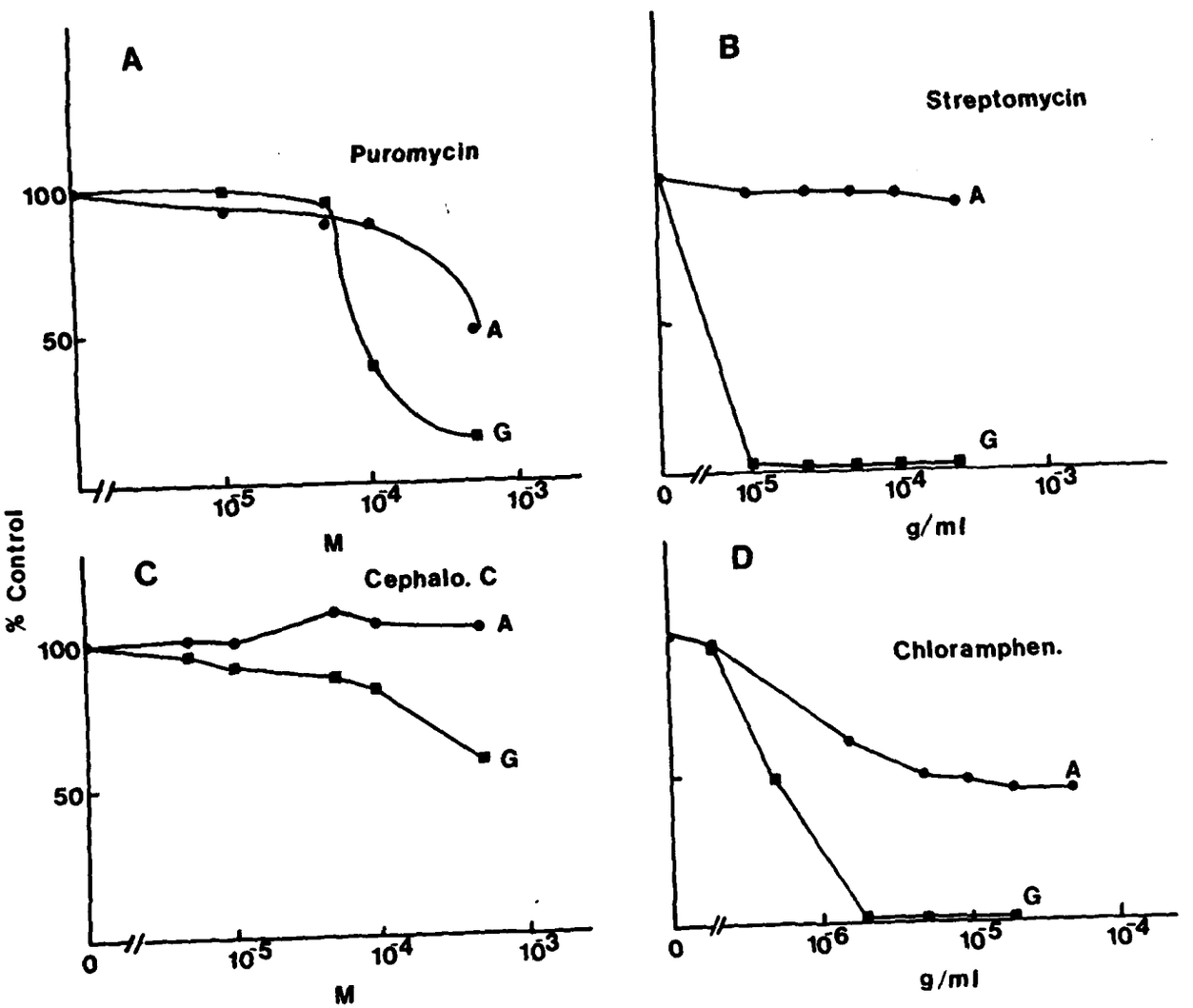
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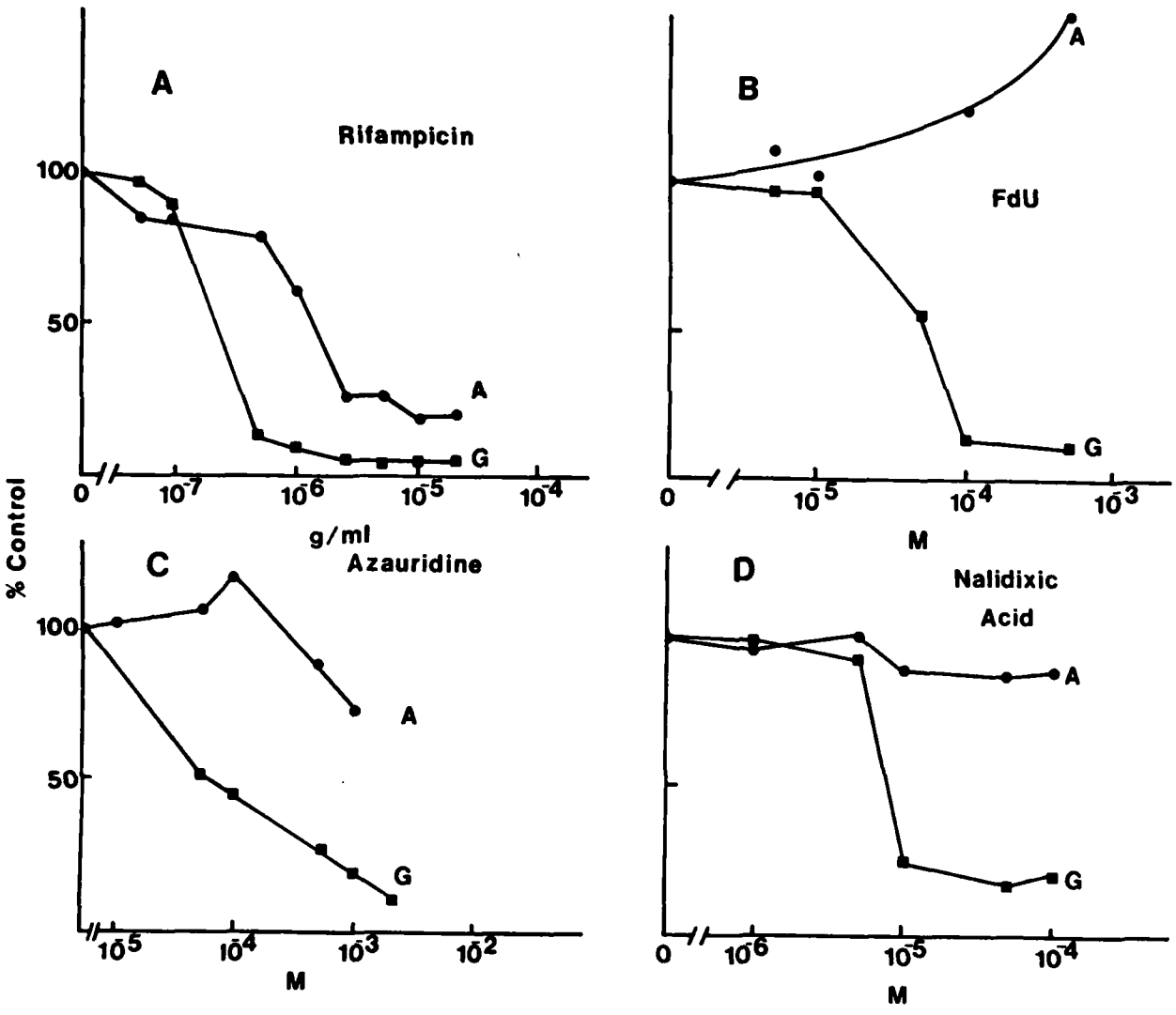
Fig 3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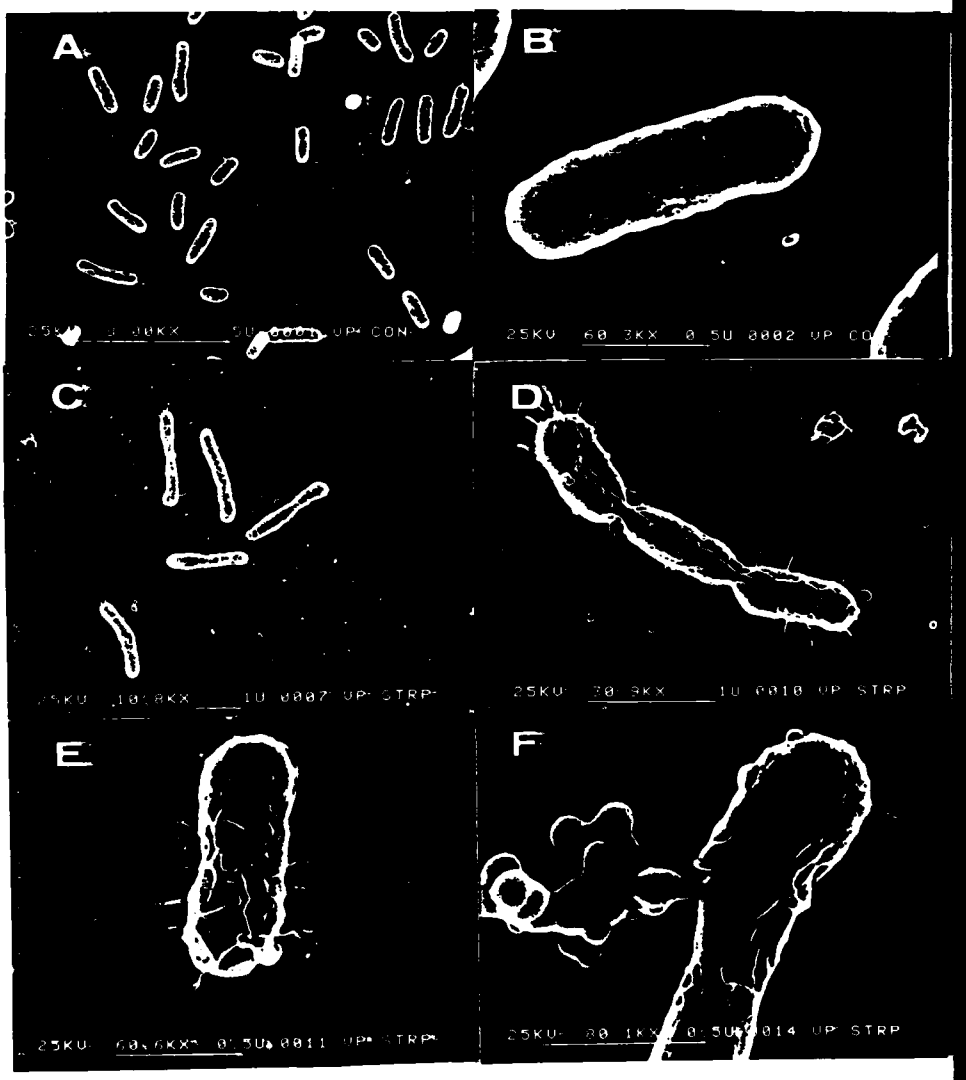
streptomycin, cell morphology was distorted and the cell surface



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Improved Microfouling Assay Employing a DNA-Specific Fluorochrome and Polystyrene as Substratum

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With a direct count assay, 10 fouling bacterial isolates have been characterized for their ability to adhere to glass cover slips and polystyrene dishes. Although most adhered in greater numbers to polystyrene, the preference was statistically significant for only seven isolates at the 95% confidence level, due in part to the greater variability in cell attachment to glass (coefficient of variation, 32.3% for glass compared with 10.0% for polystyrene). Employing polystyrene dishes, a novel microfouling assay was developed, based on the extraction and fluorometric determination of DNA. The assay was rapid, enabled the detection of as little as 0.15 μg of DNA per dish (~5,000 cells per mm^2), and showed good agreement with the direct count assay. The DNA method resulted in less variability among three replicates (average coefficient of variation, 7.06%) and allowed for estimation of bacterial density over a larger surface area per sample (1.89×10^3 mm^2) than was feasible with epifluorescence microscopy (0.06 to 0.1 mm^2).

The study of bacterial attachment to surfaces has implication in many diverse fields, including microbial ecology (16, 21), oral hygiene and dental pathology (29), enteropathology (23), urology (14), energy conversion engineering (1, 24), hydrodynamics (26), and materials deterioration research (15). Methods to enumerate bacteria attached to surfaces have been equally diverse. Counting by use of phase-contrast microscopy (19), epifluorescence microscopy (15, 31), or scanning electron microscopy (5, 8) yields additional information (distribution and morphology) of the attached population. However, these techniques are usually tedious, and scanning electron microscopy has been criticized additionally for artifacts induced during desiccation. Enumeration by plate counts (1, 7) is hampered by the selectivity of the medium employed and the subsequent underestimation of the microbial population. Optical density measurements of nonspecifically stained, attached microbial populations (10, 33) require a transparent subtratum and measure attached nonbacterial particulates (silts and clay) and stained extracellular material as well as attached bacteria.

Light section microscopy (25) and ultrasonic interferometry (27) have been employed to determine the thickness and acoustic attenuation, respectively, of mixed microbial slimes. Directed at measuring the physical properties of microfouling communities, these techniques do not yield information on the composition or total numbers of microorganisms present.

Radioisotopically labeled bacteria have been employed to study bacterial absorption to hydrophobic chromatographic supports (6) and bacterial adherence to rat bladders (14). These techniques have been found to be quite sensitive.

A variety of cellular constituents have been measured to determine the biomass of natural bacterial populations attached to surfaces, including lipids and lipid phosphate (30), ATP, protein, carbohydrate, total organic carbon and nitrogen (1), and lipopolysaccharide (9). The lipopolysaccharide technique is preferred, varying less than the others in sensitivity, specificity, and proportionality to cell number or biomass. The measurement of lipids and fatty acids can yield additional information on the composition and structure of the microfouling community (30).

Our research efforts called for the development of a rapid, sensitive, and inexpensive technique for the enumeration of bacterial isolates adhered to surfaces. In this paper we describe a microfouling assay based on the extraction and fluorometric determination of DNA. We also describe the preferences for attachment to glass (a hydrophilic surface) or polystyrene (a hydrophobic surface) of several estuarine and marine bacterial isolates.

MATERIALS AND METHODS

Isolation of fouling bacteria. Fouling bacteria were collected by a 2-h exposure of sterile 24- by 24-mm glass cover slips, polyvinyl chloride cover slips (Fisher

Scientific Co., Pittsburgh, Pa.), or Teflon squares at a depth of 1 m off a dock in the Chesapeake Bay at Chesapeake Beach, Md. These substrata were rinsed with sterile marine medium 2216 (Difco Laboratories, Detroit, Mich.) diluted 1:3 with distilled water (termed 2216/3) and either incubated in 2216/3 broth or placed on the surface of 2216/3 agar and incubated for 48 h at 15°C.

Taxonomic identification of the isolates has not been performed, with the exception of isolates 8 and 9, which have been tentatively identified as *Vibrio proteolytica* and *Alteromonas citrea* by the American Type Culture Collection. The *V. proteolytica* resembles *Aeromonas hydrophila* susp. *proteolytica*, but differs in the ability to utilize histidine and arginine as carbon sources and lacks lysine decarboxylase activity (4). Organism S was isolated from surfaces incubated in an aquarium containing Instant Ocean (Aquarium Systems Inc., Wycliffe, Ohio) and bryozoan larvae, *Bugula neretina*. The marine *Pseudomonas* sp. (National Collection of Marine Bacteria [NCMB] 2021) was a gift of Madilyn Fletcher, Environmental Sciences Department, University of Warwick, Coventry, United Kingdom.

Media and maintenance of cultures. All isolates were maintained on ASWJP (salinity [‰] ~ 10 to 11 ppt) agar medium as previously described (31), except organism S and the *Pseudomonas* sp., which were maintained on triple-strength ASWJP (salinity [‰] ~ 30.5 ppt).

Attachment assays. Growth of cells for attachment assays and the techniques for attachment assays employing epifluorescence microscopy were performed at 15°C as described previously (31). Glass cover slips for attachment assays were first detergent washed with Alconox, rinsed with tap water, and then soaked in hot nitric acid for 2 h. Cover slips were then rinsed exhaustively with tap water followed by distilled water. Since detergent washing of polystyrene dishes did not improve the results of attachment assays, dishes were used directly as supplied by manufacturer.

For attachment assays employing the fluorometric DNA technique, cells were grown overnight at 15°C on a gyratory shaker (set at 141 rpm) to an optical density corresponding to 280 to 310 on a Klett-Summerson colorimeter. Cells were harvested and washed twice with the appropriate mineral salts medium (either ASWJP for isolates 1 through 9 or triple-strength ASWJP for organism S and the *Pseudomonas* sp; all wash media lacked peptone and yeast extract) and suspended to the original growth volume in mineral salt medium. Cell concentration was determined by direct counts (31). Samples of this culture were added to sterile 60- to 15-mm hydrophobic polystyrene dishes (Falcon Plastics, Oxnard, Calif.) that contained the appropriate volumes of the mineral salt medium to obtain cell concentrations of 1.35×10^5 to 7.4×10^8 in a final volume of 8 ml. Dishes were covered and incubated statically at 15°C for 10 min to 4 h. At the termination of the incubation (attachment) period, dishes were washed by dipping four times in three baths of the mineral salts medium to remove cells. The vertical side walls of the dishes were wiped with a cotton-tipped probe to remove bacteria attached to the side walls. Four microliters of 5% (vol/vol) Triton X-100 was added to each dish, and the dish was placed on a bed of crushed ice. Four milliliters of ice-cold SSC (0.154 M NaCl, 0.015 M trisodium citrate, pH 7.0) was added to each dish, and the attached cells were broken by sonication for 45 s at 100 W with a Biosonik III sonicator (Bronwill Scientific Inc., Rochester, N.Y.). It was important to rotate the dish during the sonication period so that all cells were fully exposed to the sonic energy. We have previously shown that sonication for this length of time and at this energy is sufficient to break the organisms employed in this study and natural populations of aquatic bacteria (32). However, longer periods of sonication may be necessary to break more resistant organisms (i.e., gram-positive organisms and spores).

Two milliliters of the extract liquid was added to 1 ml of either 1.5×10^{-6} M (for 0.5 to 10 μg of DNA) or 1.5×10^{-7} M (for 50 to 1,500 ng of DNA) Hoechst 33258 (Calbiochem-Behring Corp., La Jolla, Calif.) in SSC. DNA was determined fluorometrically with calf thymus DNA standards as previously described (32).

RESULTS

All organisms isolated from surfaces exposed for 2 h to Chesapeake Bay water were short, flagellated rods (organisms 1 through 9), whereas organism S was a small, coccoid form. Table 1 shows the attachment of these isolates to glass or polystyrene. Of the isolates examined, 9 of 10 adhered in numerically greater amounts to polystyrene, although the preference was significant for only 7 ($\alpha = 0.05$; 36). There was a greater variability between replicates among glass samples, resulting in a significantly greater average coefficient of variation (32.3%) compared with polystyrene (coefficient of variation, 10%; $0.001 \leq P \leq 0.002$; $n = 20$). There was also greater variability in the number of cells attached from field to field when observed under epifluorescence microscopy for glass compared with polystyrene.

Figures 1 and 2 show the results of attachment assays on polystyrene performed by direct counts or by the fluorometric determination of DNA. In these experiments three replicates per treatment (either cell concentration or sampling time) were processed for direct counts and DNA content. For direct counts, 10 to 20 fields were counted (minimum 400 cells) representing a total area of 0.06 to 0.1 mm^2 per dish. For DNA determinations, the DNA content was determined for bacteria covering the whole dish, an area of 1.89×10^3 mm^2.

Figure 1A shows bacterial attachment as a function of supernatant bacterial concentration at high bacterial concentrations. There was a linear increase in numbers of cells attached with increasing cell concentration until the surface was saturated (confluent layer). Cells were in contact with each other, and many were attached by the bacterial pole at the highest cell concentrations. The relationship between cell concentration and the number of cells attached was linear over at least 2 orders of magnitude, as

TABLE 1. Attachment of bacterial strains to glass or polystyrene[a]

Organism	Substratum[b]	Cell density[c] (10^5 cells per mm^2 ± SD)	Coefficient of variation (%)	Significance
1	G	2.46 ± 0.32	13.0	
	P	2.91 ± 0.18	6.2	+
3	G	0.025 ± 0.006	24.0	
	P	2.5 ± 0.2	8.0	+
4	G	0.028 ± 0.005	17.9	
	P	0.045 ± 0.005	11.1	+
5	G	1.52 ± 0.58	38.2	
	P	3.05 ± 0.078	2.6	+
6	G	0.82 ± 0.27	33.0	
	P	0.69 ± 0.25	36.2	
7	G	0.037 ± 0.024	64.9	
	P	0.081 ± 0.015	18.5	
8 (*V. proteolytica*)	G	2.14 ± 0.55	25.7	
	P	3.82 ± 0.086	2.3	+
9 (*A. citrea*)	G	1.80 ± 0.52	28.9	
	P	2.52 ± 0.265	10.5	+
Organism S	G	1.35 ± 0.49	36.3	
	P	2.14 ± 0.078	36.4	
Pseudomonas sp. strain NCMB 2021	G	0.033 ± 0.014	42.4	
	P	0.178 ± 0.002	1.12	+
	PLP	0.026 ± 0.008	30.8	

[a] Cells were grown to an initial cell density corresponding to an optical density of 260 on a Klett-Summerson colorimeter. Cells were washed twice and resuspended in the same volume used for growth. Four milliliters of culture medium was added to 4 ml of mineral salt medium in a polystyrene dish with or without a 24- by 40-mm cover glass and incubated for 4 h at 15°C.

[b] Substrata were glass coverslips (G), polystyrene dishes (P), or plasma-cleaned polystyrene dishes (PLP).

[c] Means of three replicate surfaces.

seen at low bacterial concentrations in Fig. 1B. The sensitivity of this technique was determined to be 150 ng of DNA per dish, which corresponds to less than 5,000 cells per mm^2.

The attachment of *Vibrio proteolytica* as a function of time appears in Fig. 2. Again there was relatively good agreement between direct counts and DNA. Curiously, neither curve could be extrapolated through the origin. Direct counts of the early samples (10 to 15 min) were obtained with difficulty due to the patchiness of cells in these samples.

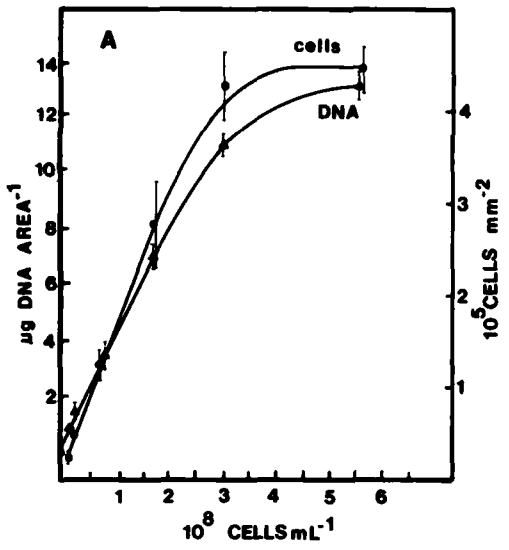
Figure 3 shows the relationship between cells per unit area and DNA per unit area for 44 determinations (each is the mean of three replicates) from 10 experiments (different lots of cells). The correlation coefficient of the linear regression, r (36), is 0.99, and the slope is 13.74×10^{-15} g of DNA per cell, with a coefficient of variation, Sb/b (36), of 12.6%. The scatter within the plot is typical for cellular DNA determinations for different lots of cells (22).

The average coefficient of variation for all data sets (44 determinations, three replicates each) for the direct count technique on polystyrene was significantly greater ($0.01 \leq P \leq 0.02$, t test [36]) than for DNA determinations (10.2% compared with 7.06%).

DISCUSSION

A simplified microfouling assay has been developed that is based on the fluorometric determination of DNA in attached bacterial cells. Results obtained with this technique agree well with those obtained by direct count employing epifluorescence microscopy. The advantages of the DNA technique include ease in handling large numbers of samples (30 samples can be conveniently assayed in less than 2 h), better replication (smaller coefficients of variation), and less investigator bias (for example, one technician consistently counted 10 to 20% more cells than another in any one microscopic field). The specificity and quantitative nature of the interaction of Hoechst 33258 with DNA has been described elsewhere (32).

It should be emphasized that direct counting techniques based on epifluorescence microscopy are estimates of bacterial number and not biomass. DNA content of bacterial populations had been shown to correlate well with bacterial cell number, whereas the relationship between DNA content and bacterial biomass is less well defined. Torsvik and Gokskoyr (35) measured DNA by two fluorometric techniques in soil fractionated to remove organisms other than



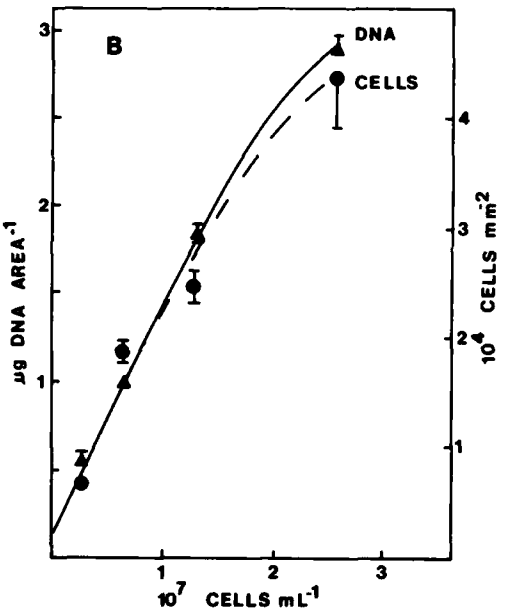


FIG. 1. Attachment of *V. proteolytica* to polystyrene at high cell concentrations (A) or low cell concentrations (B). Attached cell density was determined by direct counts (●) or estimated by total DNA content (▲) of cells attached to the dish surface ($1.89 \times 10^3/mm^2$) in Hoechst 33258 at a final concentration of 5×10^{-7} M (A) or 5×10^{-8} M (B).

bacteria and found a good correlation between DNA content and bacterial cell number. We have previously found good correlations between DNA content and cell number for bacterial cultures and natural microbial populations (32; J. H. Paul and D. J. Carlson, submitted for publication). This may be due to the relatively constant size of bacterial genomes (2, 20).

Holm-Hansen (17) found a correlation between cell size and DNA content in various eukaryotic microalgae. Cellular DNA content was correlated to organic carbon content since algal DNA content varies among different-sized species by several orders of magnitude (17). Breter et al. (3) also proposed a relationship between biomass and DNA content. However, in studies of natural phytoplankton populations, Holm-Hansen (18) found DNA to be a poor estimate of biomass or particulate organic carbon.

The general preference of marine fouling bacterial isolates for hydrophobic surfaces (found in this study) has also been found by others (12, 13). Cell surface hydrophobicity has been implicated as an important factor in the interaction of bacteria with various interfaces (6, 28, 34). The greater variability observed in the numbers of cells attached to glass may be due to inhomogeneities and inconsistencies in the glass surface or the inability to truly clean glass, a reactive substance. Polystyrene is presently employed in our studies on the physiology of bacterial attachment due to the greater reproducibility.

Interestingly, this preference for hydrophobic surfaces may not occur in natural populations of marine bacteria. For example, Dexter et al. (9) found that the number of bacteria attached to glass in Woods Hole Harbor exceeded that attached to polystyrene (and other hydrophobic, low-energy surfaces) by 1 to 3 orders of magni-

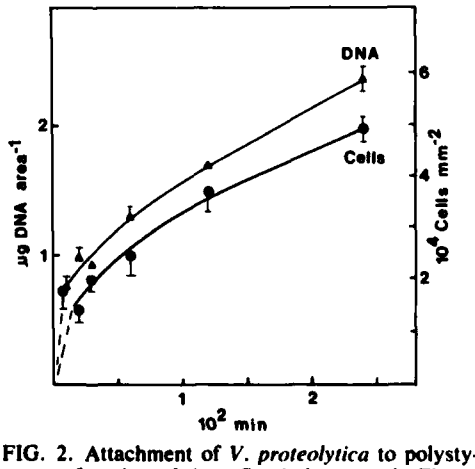


FIG. 2. Attachment of *V. proteolytica* to polystyrene as a function of time. Symbols are as in Fig. 1. The initial cell concentration was 2.43×10^7 cells per ml. The dashed line indicates extrapolation through the origin.

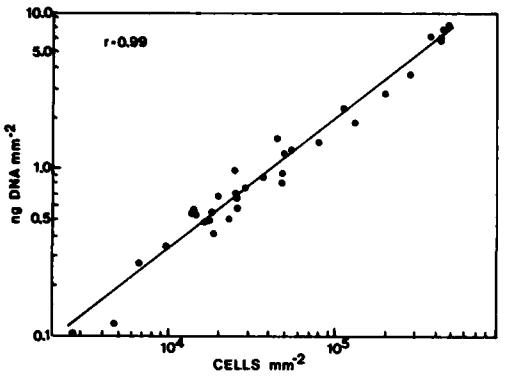


FIG. 3. Relationship between DNA content per unit area and number of attached cells for *V. proteolytica*. Each point is the mean of three determinations.

tude. The reasons for the discrepancies observed between bacterial isolates and natural populations of bacteria remain unclear.

Curves for the rate of bacterial attachment for *Vibrio protolytica* strongly resemble those obtained by Fletcher (11) for *Pseudomonas* sp. NCMB 2021 in that extrapolation of the curve does not result in intersection with the origin. This suggests that the rate of attachment is biphasic, with a very rapid rate of attachment occurring in the first 10 to 30 min, followed by a slower, more linear rate after 30 min. The meaning of these biphiphasic kinetics is not clear, but may suggest the involvement of two temporarily separate processes.

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